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Award Number: DAMD17-97-1-7248

TITLE: Molecular Mechanisms of Dietary Fatty Acids on Breast
Cancer Growth and Development

PRINCIPAL INVESTIGATOR: Michael W. Kilgore, Ph.D.

CONTRACTING ORGANIZATION: Clemson University
Clemson, South Carolina 29634-5702

REPORT DATE: January 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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20010122 133

REPORT DOCUMENTATION PAGE

*Form Approved
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1. AGENCY USE ONLY (Leave blank)			2. REPORT DATE January 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Jan 99 - 31 Dec 99)
4. TITLE AND SUBTITLE Molecular Mechanisms of Dietary Fatty Acids on Breast Cancer Growth and Development			5. FUNDING NUMBERS DAMD17-97-1-7248	
6. AUTHOR(S) Michael W. Kilgore, Ph.D.			8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Clemson University Clemson, South Carolina 29634-5702				
E-MAIL: mkilger@clemson.edu			9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
10. SPONSORING / MONITORING AGENCY REPORT NUMBER				
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)				
<p>The link between diet and breast cancer has been long postulated and recently, extensively investigated. Identifying a molecular mechanism linking diet and breast cancer, however, has remained elusive. Recently, we identified an isoform of the cellular receptor called the peroxisomal proliferator-activated receptor (PPAR) in several human breast cancer cell lines. Activation of PPAR can alternatively lead to tumor induction or differentiation into a more benign state, depending on the tissue and the isoform expressed. Activators of this receptor are a group of structurally diverse compounds that include the fibrate class of hypolipidemic drugs, phthalate plasticizers and herbicides. Recently, it has been shown that this receptor is capable of binding and being activated directly by polyunsaturated fatty acids (PUFA). We have demonstrated that human breast cancer cell lines express PPARγ1 and that individual fatty acids are capable of functioning as selective agonist or antagonist of PPAR action. Furthermore, we present evidence that the signal transduction of PPAR can be mediated by the presence of the estrogen receptor. Finally, we have verified the fidelity of our transcriptional reporter system using antisense expression vectors in transient transfection analysis. These studies may lead to better understanding of the risk of specific dietary components. The data presented in this report make it clear that fatty acids can indeed function as hormones and could lead to important information regarding dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and</p> <p>management of this devastating disease.</p>				
14. SUBJECT TERMS Breast Cancer, diet, fatty acids, Estrogen receptor				15. NUMBER OF PAGES 51
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

*Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102*

FOREWORD

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In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Michael Kilgore 1.27.2000
PI - Signature Date

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INTRODUCTION

The link between diet and cancer has been long postulated and recently, extensively investigated. Identifying a molecular mechanism linking diet and breast cancer, however, has remained elusive. Recently, we identified the cellular receptor called the peroxisomal proliferator-activated receptor (PPAR) in human breast cancer cells. PPAR is capable of binding and being activated directly by a variety of ligands including polyunsaturated fatty-acids (PUFA). In order to elucidate the functional significance of our findings, we have set about to determine the form(s) of PPAR and its heterodimeric partner, the retinoid X receptor (RXR), expressed in human breast cancer cells and to examine the regulation of PPAR expression in MCF-7 and MDA-MB-231 cells. The long-term goal of this work is to determine whether PPAR expression and activation is important in the etiology of breast cancer. To this end, we are examining the isoforms expressed, the regulation of expression and the molecular mechanism of transcriptional activation of PPAR using cell culture systems. These data could lead to new approaches in the treatment and prevention of breast cancer through the development of novel therapeutic interventions and revised dietary guidelines for women at risk of breast cancer as well as breast cancer survivors.

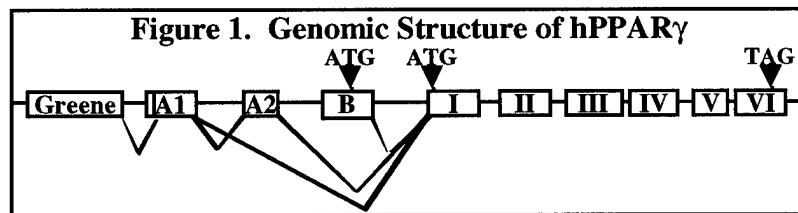
Body

Description of Training:

To date three students have received their masters' degrees (MS) as a result of this award. Ms Sudha Thoennes is currently a technician at the University of Florida, Gainesville Medical School and is investigating viral mechanisms of oncogenesis. Mr. Eiichi Sengoku is currently Product Manager for North American Operations at Nalgen in Rochester New York. Major Mark Corbett is serving in the US Army in the Chemical Corp following completion of his degree. Ms. Rupalika Singh, an undergraduate student, has worked in the lab for the last 2 semesters has been accepted to medical school in part based upon her research experience gained in the PI's lab as a consequence of this grant support. Dr. Xin Wang has been in the lab as a postdoctoral fellow since October 1998 and has already presented an abstract at one international conference. She continues to progress well and is currently writing a manuscript which will be submitted this spring. Ms. Susan Pierce is a Ph.D. candidate in the Biochemistry program and Ms. Evelina Loghin is a master's candidate who is currently scheduled to graduate in December 2000.

Research Accomplishments:

PPAR has three known members, α , β (also termed δ) and γ and within gamma, 3 more subtypes have been identified, γ_1 , γ_2 and γ_3 (1-3). Using a mouse cDNA we originally determined that PPAR is expressed in both MCF-7 and T-47-D human breast cancer cells by Northern blot analysis. To confirm this observation and to identify the form(s) of PPAR expressed a cDNA library was constructed from MCF-7 cells and screened with both PPAR and RXR probes. These were designed against the DNA binding domain of the receptors, the most highly conserved portion of the gene, to identify all forms present. The isolated C1 clone was clearly PPAR γ , containing the ATG start site at base +173 (Accession # L40904) and the stop sign at base 1607 which encodes the full open reading frame of the 478 amino acid protein (4). Our sequence diverged from that reported by Greene and co-workers immediately 5' of the start site of translation. An additional 74 bases were inserted between the untranslated exon A1 and the ATG-containing exon (exon I). This was later described as exon A2 (2). As seen in figure 1, exon A2 is either a splice variant of γ_1 expression or can be the first untranslated exon and may be associated with unique promoter elements (3). PPAR γ_1 and 3 code



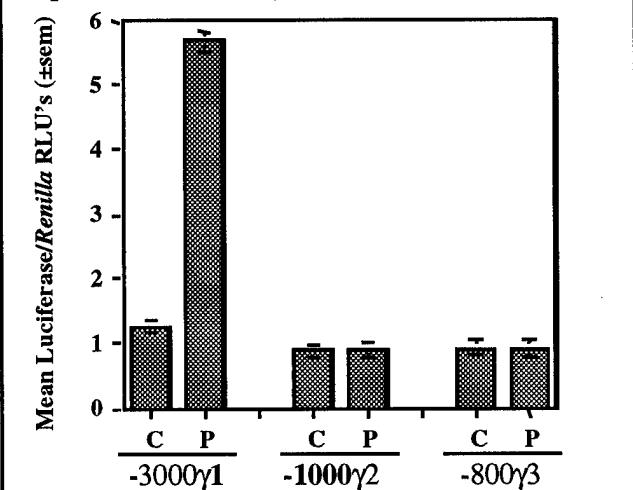
for identical proteins while γ_2 contains an additional 28 N'-terminal amino acids. The functional significance of these differences remain unclear. None of the cDNA's we have isolated contains exon B which encodes these additional amino acids and RT-PCR using an exon B-specific oligonucleotide could detect no exon B usage in MCF-7 or MDA-MB-231 cells. For these reasons, we believe the only protein expressed in these cells is PPAR γ_1 . From the RT-PCR data some transcripts contain A1+A2 spliced onto exon I while about half contain A1 fused directly to exon I.

Since the genomic structure and thus the regulation of PPAR is complex, we sought to determine if other forms of PPAR are expressed in MDA-MB-231 cells which have not been previously reported. To this end we have performed 5' RACE (rapid amplification of cDNA ends). Thus far we have isolated 4 clones which are depicted in figure 2. In the top structure exon A1 is fused to A2, then exon I, similar to previous reports (2). The second clone contains A2 fused to exon I but contains more than 180 bases of the 5' flanking sequence of A2 previously reported to be $\gamma 3$ promoter sequence (3). Since the RACE library was constructed from total RNA and the distance between exon's A2 and I are more than 20kb apart, it is very unlikely this represents contamination from genomic DNA. Thus it remains possible that transcriptional initiation in MDA-MB-231 cells lies further 5' of the transcriptional start site of A2 in adipocytes (3). The third clone contains an exon reported only in Rhesus monkeys (AF033342) fused to A2 and exon I (5). The human sequence we have cloned is 96% identical to that of the Macaque. The fourth clone we isolated is unique and has not been previously reported to Genebank. Currently the relative levels of expression driven from each promoter, the genomic structure of this gene and the functional significance of these findings are still unclear and the subject of further investigation. We have obtained three genomic reporter constructs which drive the tissue-specific expression of PPAR in adipocytes and hepatocytes specific for $\gamma 1$, 2 and 3 (2,3). These contain 3000 bp upstream of A1, 1000 bp upstream of B and 800 bp upstream of A2, respectively, driving the expression of luciferase (a kind gift from Dr. Johan Auwerx, Institut de Genetique et Biologie Moleculaire et Cellulaire, Illkirch, France). These were transfected into both MCF-7 and MDA-MB-231 cells and relative luciferase activity compared to control plasmids containing the basic luciferase vector (Promega) lacking the 5' flanking sequences. The data shown in figure 3 is typical of what is seen in both cell lines. These data indicate that the 3000 bp flanking the 5' of exon A1 and not the elements upstream of B or A2, contains *cis*-regulatory elements capable of mediating basal expression of PPAR in both MCF-7 and MDA-MB-231.

Figure 2. RACE Clones Isolated from MDA-MB-231 Cells

A1	A2	Exon I
5' flanking	A2	Exon I
AF033342	A2	Exon I
Unknown	A2	Exon I

Figure 3. Promoter Activity of the 5' Flanking Sequences of PPAR $\gamma 1$, $\gamma 2$ and $\gamma 3$ in MCF-7 Cells



In contrast to the complex pattern of PPAR expression, a single cDNA species of RXR was isolated from both the MCF-7 and the MDA-MB-231 libraries. Despite using a degenerate probe that would have hybridized to all forms of RXR under the conditions used in Southern blot analysis, the only cDNA identified was RXR α . A full-length cDNA was isolated and contained no differences from those reported previously (6).

To examine the functional responsiveness of PPAR in breast cancer cells we have constructed transactional reporters containing peroxisome proliferator response elements (PPRE). These have been used in transient transfection assays to assess the transactivation of the PPAR-RXR complex. We have demonstrated that synthetic peroxisome proliferators increase the activity of transcriptional reporters containing either the peroxisome proliferator response element (PPRE) or the perfect direct repeat (DR-1) response element (7). Furthermore, we have gone on to demonstrate that individual fatty acids are capable of selectively functioning as agonists or antagonist of PPAR (see attached manuscript in press, MCE, 2000). This observation touches the central questions regarding whether PPAR mediates the effects of individual fatty acids present in diets and alters the incidence or growth rates of breast tumors. These data also indicate that PPAR is constitutively transcribed, translated and transactivated since treatment with ω -3 fatty acids reduced reporter activity to levels below control. Furthermore, transactivation of PPAR with fatty acids correlates with proliferation *in vitro*. It is not clear, however, whether this is a direct or indirect effect. To prove that the transcriptional reporter is indeed measuring the transactivation of PPAR we have constructed a PPAR γ antisense expression vector in order to block translation of the mRNA and thus reduce the basal levels of the protein. The antisense expression vector codes for an antisense mRNA spanning the sequence between the start of translation at base 173 and base 693 (accession # L40904). As seen in figure 4, the expression of the antisense mRNA selectively inhibited reporter activation while the sense vector had no effect (data not shown). Furthermore, the antisense expression not only inhibited activation of PPAR but it also inhibited basal transactivation again indicating that PPAR is constitutively transactivated in these cells.

Since many breast tumors are initially estrogen receptor (ER) positive and become ER negative, we have examined the transactivation of PPAR in cells which do or do not express the ER. MDA-MB-231 cells which do not express ER, were transfected with expression vectors for ER α or ER β and the PPRE-mediated reporter. As seen in figure 5, the mere presence of the ER inhibited PPAR transactivation in a ligand independent manner. This observation suggests that signal cross-talk exists between ER and PPAR. Using gel shift analysis, this interaction does not appear to be due to

Figure 4. Antisense PPAR γ inhibition of PPRE-Mediated Transcription in Transfected MCF-7 Breast Cancer Cells

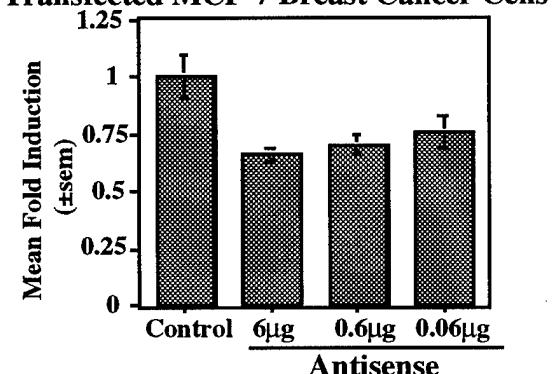
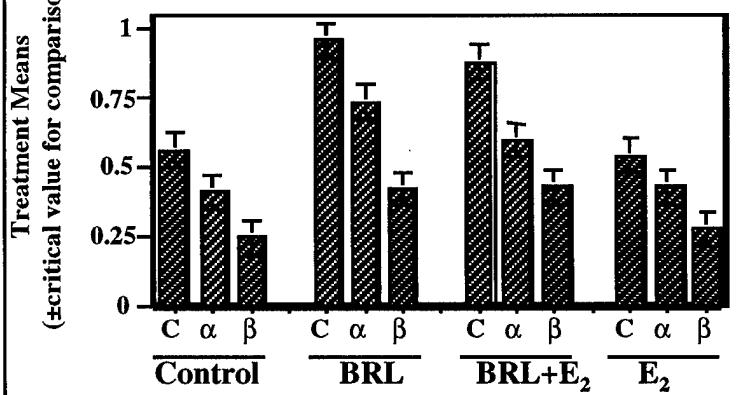


Fig. 5. PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ER α or ER β



competition for DNA binding (data not shown). This observation could have important implications for breast cancer treatment as the use of antiestrogen such as Tamoxifen is widely employed. The functional significance of this observation and the molecular mechanisms of this effect are under investigation.

In summary we have made significant progress toward all three specific aims outlined in the grant proposal. As stated in aim 1, we have determined the isoforms of both PPAR and RXR and have found new complexity in the regulation of PPAR which could have implications in the tissue-specific control of expression. Toward understanding the regulation of PPAR expression, aim 2, we have demonstrated that there is a significant level of basal PPAR expression in both MCF-7 and MDA-MB-231 cells. Furthermore, the sequence flanking the 5' start site of Exon A1 mediates basal PPAR expression in these cells. We are further defining this region to idnetify the *cis* regulatory elements responsible for this regulation. In defining the molecular mechanism of transactivation of PPAR, aim 3, we have identified a unique form of regulation which involves signal cross talk between PPAR and the estrogen receptor. The mechanism of this unexpected yet potentially important effect and the functional significance are currently under investigation. Clearly PPAR γ 1 is present in breast cancer cells and may provide a direct link between diet and the increased rate of breast cancers seen in this country. These studies may lead to better understanding of the risk of specific dietary components. The data make it clear that fatty acids can indeed function as hormones and this information could lead to important new discoveries impacting dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.

Methods

Cell Culture: MB-MDA-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscripts.

Probe Construction: The mouse pSV-Sport/PPAR γ 2 plasmid was received as a kind gift from Dr. Bruce M. Spiegelman's lab at the Dana Farber Cancer Institute. The hRXR α expression vector (pCMV-RXR α) was a kind gift from Dr. Ron Evans (Howard Hughes Medical Institute, San Diego, CA). Primers were designed to each which aplified a 288 bp and a 321 bp fragment of the Dna binding domain of PPAR and RXR, respectively. The reaction utilized 40 ng of plasmid, 20 pmol of each primer, dNTP (10 mM each) at 0.2 mM, 1.5 mM MgCl₂, 1X final concentration polymerase buffer (Promega), and 2.5 units *Taq* Polymerase.

cDNA Library Construction: Using oligo dT mRNA a cDNA library was created using the Time Saver synthesis kit (Pharmacia) as per instructions. After the second strand synthesis, 5 units of T4 RNA Polymerase were added to the reaction for 1 h at 37°C. The cDNA was then ligated and packaged into the Lambda Zap Express vector (Stratagene) as per instructions. The library was titered at 1.3 x 10⁸ plaque forming units/ml (PFU/ml) with ≤15% non-recombinants.

Northern Blot Analysis: Please see attached manuscript, Kilgore *et al.*, MCE.

Transfection and proliferation: Please see attached manuscript, Thoennes *et al.*, In press.

Library Screening: A total of 2×10^5 PFU were lifted onto Hybond N+ membranes, denatured, UV crosslinked with 12,000 μ J (Stratagene) and air-dried. Membranes were probed following prehybridization and hybridization steps were performed twice in 2X SSC for 20 min each wash with a final wash in 1X SSC for 20 min. All washes were performed at 65°C. Membranes were then put under Hyperfilm (Amersham) and developed. Dilutions of the phage plugs were then used to plate out for the next round of screening. Third or fourth round of plating was performed to yield a single population of phage. Helper phage plasmid excision was performed as per instructions that resulted in a pBK-CMV plasmid with cDNA insert.

Sequencing: Sequencing reactions consisted of 400 ng plasmid DNA, 8 μ l of ABI Prism Ready Reaction mix (Perkin-Elmer/ABI), 5 pmol of primer, sterile distilled water to a total reaction volume of 20 μ l and an oil overlay. Sequencing reactions were run on a ABI 373 Stretch sequencer (Applied Biosystems, Inc.).

RACE and RT-PCR: Single stranded cDNA was synthesized by MMLV reverse transcriptase from polyA⁺ RNA (Clontech). For the initial reaction an oligonucleotide was used at bases +221 to +243 at the 3' end of exon I. In the PCR phase of both reactions the 3' oligonucleotide was between bases +198 to +218 for increased specificity of the PCR reaction. In the RT-PCR experiments primer HPR4 (438-419 of L40904) or HPR4 -10 (428-409) (both are contained within exon II). For the 4 sets of PCR reactions, 20 pmole of each of four 5' primers were used: "5'Greene" (which starts at bp23 of L40904), A1 (starting at bp 95 of L40904), A2 (starting at bp 172) and B (starting at bp 95, (2)). PCR products were ligated into a TA 2.1 cloning vector (Invitrogen) and screened using the BPF1 probe (bp 199-216). Eleven positive clones from the A2 primed population, and eleven clones from the A1 primed population, were identified and sequenced.

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7. Kilgore, M. W., Tate, P. L., Rai, S., Sengoku, E., and Price, T. M. (1997) *Mol Cell Endocrinol* 129(2), 229-35

Key Research Accomplishments:

- Verification of PPAR γ and RXR α expression in multiple breast cancer cell lines.
- Demonstration of a functional PPAR response in MCF-7 and MDA-MB-231 cells.
- Demonstration that fatty acid selectively function as agonist or antagonist of PPAR transactivation.
- Determined PPAR to be both constitutively transcribed and transactivated in MCF-7 and MDA-MB-231 cells.
- Identified signal cross talk between the estrogen receptor and PPAR.

Reportable Outcomes:

Manuscripts:

- **MW Kilgore, PL Tate, S Rai, E Sengoku and TM Price** 1997 MCF-7 and T47D Human Breast Cancer Cells Contain a Functional Peroxisomal Response. *Molecular and Cellular Endocrinology*, 129:229-235.
- SR Theonnes, PL Tate, TM Price and **MW Kilgore** 2000 Differential Transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells.. (in press, *Molecular and Cellular Endocrinology*).
- X Wang and **MW Kilgore** 2000 Signal cross-talk between the alpha and beta forms of the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (in preparation).

News Releases:

- Featured on WYFF ABC affiliate local news release as area breast cancer related research funded by the US Army.
- Featured article in The Greenville News highlighting the contributions made to the area's research supported by The US Army.

Abstracts Presented:

1. E Sengoku and **MW Kilgore**. MCF-7 cells express a unique form of the peroxisome proliferator-activated receptor. Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.
2. S Rai and **MW Kilgore**. Selective activation of the peroxisome proliferator-activated receptor by dietary fatty acids in human breast cancer cells. Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.
3. S Rai, PL Tate, TM Price, **MW Kilgore**. Role of dietary fats in PPAR-mediated carcinoma in MCF-7 human breast cancer cell lines. (80th Annual meeting of the Endocrine Society, New Orleans, LA, June 1998).
4. X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. Eighth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1999.
5. X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (81st Annual meeting of the Endocrine Society, San Diego, CA, June 1999).
6. E. Loghin and **MW Kilgore**. Regulation of peroxisome proliferator-activated receptor gamma (PPAR γ) gene expression in MCF-7 and MDA-MB-231 human breast cancer cells. 9th Annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January 2000.
7. Susan Peirce and **Michael W. Kilgore**. A Dominant Negative Mutant of PPAR γ Reduces PPRE-mediated Reporter Activity in MCF-7 Human Breast Cancer Cells. 9th Annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January 2000.

Invited Lectures:

1. Effects of Peroxisome Proliferators in the Human Breast: A Molecular Model for the Role of Diet in Breast Cancer. Carolinas Medical Center. Charlotte, NC, March 1998.
2. Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. University of Texas Health Center at Tyler. January 1999.
3. Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. Clemson University Institute for Environmental Toxicology. March 1999.
4. Molecular mechanisms of nuclear receptor transactivation in human breast cancer cells by dietary fatty acids and phytoestrogens. University of Louisville School of Medicine. April 1999.
5. Molecular mechanisms of peroxisome proliferator-activated receptor transactivation in MCF-7 and MDA-MB-231 human breast cancer cells. Texas Tech University HSC. August, 1999.

Invited Lectures Continued:

6. Molecular mechanisms of nuclear receptor in human breast cancer cells by dietary acids and phytoestrogens. University of Kentucky, Chandler Medical Center, Department of Pharmacology, September, 1999.
7. Molecular mechanisms of nuclear receptor in human breast cancer cells by dietary acids and phytoestrogens. MD Anderson Cancer Center, Research Park, Department of Molecular Carcinogenesis. November, 1999.

Degrees obtained due to the support generated by DAMD17-97-1-7248:

Student	Degree Obtained	Graduation
Ms. Sudha R. Thoennes	MS	August, 1998
Mr. Eiichi Sengoku	MS	August, 1998
Major Mark T. Corbett	MS	August, 1999
Ms Rupalika Singh	BS	May, 2000

Funding Applied for:

GHS/CU Biomedical Cooperative Competitive Research Grants. 10/1/98-9/30/99. **Total cost \$68,000.**

American Institute for Cancer Research. Molecular effects of dietary fatty acids on human breast cancer cells. PI, 20% commitment. 6/1/2000-5/31/2002. **Total cost \$162,086.**

Komen Foundation, Race for the cure. Exploring the Molecular Mechanism of Ginseng's Ability to Suppress Breast Cancer Cell Growth. PI, 10% commitment. 3/1/2000-2/28/2001. **Total cost \$15,000.**

Employment based upon training supported by this award:

Student	Degree Obtained	Current Position, Location
Ms. Sudha R. Thoennes	MS	Technician, U of Florida School of Medicine
Mr. Eiichi Sengoku	MS	Product Manager for North American Operations (Nalgen), Rochester, New York
Major Mark T. Corbett	MS	US Army Chemical Corp, Washington, D.C.



Rapid paper

MCF-7 and T47D human breast cancer cells contain a functional peroxisomal response

Michael W. Kilgore ^{a,*}, Patricia L. Tate ^a, Sudha Rai ^b, Eiichi Sengoku ^b, Thomas M. Price ^a

^a Greenville Hospital/Clemson University Cooperative Research and Education Program, 132 Long Hall, Clemson University, Clemson, SC 29634–2001, USA

^b Department of Biological Sciences, 132 Long Hall, Clemson University, Clemson, SC 29634–2001, USA

Received 13 December 1996; accepted 26 February 1997

Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that regulate transcription of target genes. Since attempts have been made to correlate the ingestion of high-fat diets, itself a peroxisome proliferator, with the occurrence of breast cancer, we set about to determine if human breast cancer cells contained a functional PPAR. In this report, we demonstrate the presence of an mRNA in two breast cancer cell lines (MCF-7 and T47D) which is specifically recognized by a mouse PPAR γ 2 probe. Furthermore, in gel shift assays a consensus PPAR response element (PPRE) was specifically bound by nuclear extracts from MCF-7 cells and was further retarded by antibodies raised to mouse PPAR γ . Finally, when transfected with a PPRE-luciferase transcriptional reporter construct, transcription was increased in response to activators of PPAR and its dimeric partner the retinoic acid X receptor (RXR). These data indicate that peroxisomal proliferators are capable of mediating transcription in human breast cells and suggest the possibility of a physiological role in the breast. © 1997 Elsevier Science Ltd.

Keywords: PPAR; Peroxisomal Proliferator; MCF-7 cells; human breast cancer

1. Introduction

Peroxisome proliferators are a group of structurally diverse compounds that were characterized based on their ability to cause an increase in both the size and number of hepatic peroxisomes when administered to rodents [1,2]. These cellular changes are accompanied by a concomitant increase in their capacity to metabolize lipids due to a dramatic increase in the expression of enzymes required for β -oxidation [3]. This class of compounds include phthalate plasticizers, herbicides and the fibrate class of hypolipidemic drugs including

WY-14,643 [4]. Furthermore, peroxisome proliferation can be induced by maintaining animals on high-fat diets [5].

The identification of an orphan nuclear receptor activated by peroxisomal proliferators [3] has shed significant insight into the molecular mechanism by which these factors exert their pleiotropic effects. This receptor, termed the peroxisomal proliferator activated receptor (PPAR), is also activated by a variety of fatty acids, which results in the transcriptional regulation of multiple enzymes in the β and ω fatty acid oxidative pathways [5–9]. PPAR is a member of the steroid hormone receptor superfamily and, as such, is a ligand activated transcription factor. Peroxisomal proliferators activate transcription by binding PPAR which, in turn, can heterodimerize with the retinoic acid X receptor α (RXR α) [10]. Although the sequence of events from

* Corresponding author. Present address: GHS/CU Biomedical Cooperative, 445 Brackett Hall, Clemson University, Clemson, SC 29634–1903, USA. Tel.: +1 864 6561859; fax: +1 864 6561498; e-mail: MKilgor@Clemson.Edu

stimulation to transcriptional activation has not been delineated, the ligand activated complex is capable of binding specific DNA sequences termed peroxisomal proliferator response elements (PPRE) or DR1 sites [11,12] and participate in activating the basic transcriptional machinery resulting in the upregulation of target genes.

Several forms of PPAR have been cloned including murine PPAR α [2,3], and γ 1 and 2 [13], rat PPAR α [14], human PPAR α [15] γ 1 [16] and γ 2 [17] and *Xenopus* α , β and γ [18]. A fourth member of this family is represented by NUC1 [19]. The α , β and γ forms of PPAR have highly conserved regions in the DNA binding domain (domain C) as well as in the ligand binding domain (E). These receptor subtypes differ in chromosomal location and tissue-specific patterns of expression. Furthermore, different transcriptional targets are activated dependent upon the ligand used as well as the promoter and cell context [16,20,21]. Although a wide variety of compounds including fatty acids [5,22] and arachidonate activate various forms of PPAR's, recent data suggest that the endogenous ligand for PPAR γ to be 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (prostaglandin J₂, PGJ₂) [23,24].

Combining the facts that, (1) peroxisomal proliferators are known carcinogens acting through a nuclear transcriptional factor, (2) this receptor can be activated by dietary fatty acids and (3) some epidemiological data suggests high-fat diets are associated with an increase in breast cancer rates [25], we set about to determine whether peroxisomal proliferators might play a role in breast disease. In this report, we present data which indicates that some form of PPAR is present in MCF-7 human breast cancer cells which is capable of recognizing and binding specific response elements. Finally, stimulating these cells results in the upregulation of a transcriptional reporter containing peroxisomal response elements indicating these cells contain a complete and functional response to peroxisome proliferators.

2. Methods

2.1. Cell Culture

T47D and MCF-7 cells are routinely cultured in RPMI and Dulbecco's modified Eagle's medium (DMEM) (GibCo), respectively, lacking phenol red supplemented with 10% fetal calf serum (FCS). Cells are grown in charcoal stripped serum (CSS, HyClone) for 7 days prior to treatments which has proven useful in lowering basal expression in preliminary experiments. Starved cells are discarded following experiments and fresh cells, maintained in fetal serum, are starved each week. Cells are maintained at 37°C in 5%

CO₂ in T-75 flasks and transferred to 6-well plates (Corning) 2 days prior to transfection. Cells are always transfected at $\leq 50\%$ confluence, provided an overnight recovery and treated for 18 or 24 h.

2.2. Northern Blot Analysis

Northern blot analysis was performed on poly (A)⁺ RNA isolated from MCF-7 and T47D cells. Three hundred milligrams of total RNA were isolated from 10, 100 mm² dishes (Corning) of MCF-7 cells in a CsCl gradient as described [26]. Messenger RNA was then isolated using the PolyATtract System IV (Promega). Alternately, mRNA was isolated from T47D cells using the FastTrack isolation method (Invitrogen). Total sunflower (*Helianthus annuus* cv. Mammoth) RNA was isolated essentially as described using the LiCl method [26] from 16 day old seedlings (a kind gift from Dr Laura Georgi, Clemson University, Clemson, SC). In each case RNA was resolved at 60 V for 2 h on a formaldehyde gel and blotted overnight via capillary blot with 10 × SSC [26]. Samples were fixed to membranes (Zeta-Probe, BioRad) by ultra violet (UV) cross-linking (Stratalinker, Stratagene) and baked in a vacuum oven at 80°C for 30 min. Membranes were then prehybridized for 6 h at 65°C and hybridized with a mouse PPAR γ 2 cDNA (a kind gift from Dr BM Spiegelman, Dana-Farber Cancer Institute, Boston, MA) at 65°C overnight. The probe was a polymerase chain reaction (PCR) product labeled with [α ³²P]dCTP using the random prime method [26]. The oligonucleotides used to create the template for the probe were 5'-ATTCTGGCCCACTT3' and 5'-GCTAATA-CAAGTCCTTGT3' for the forward and reverse, respectively. Following hybridization, the membranes were washed three times at 65°C for 10 min each in 1 × SSC and 0.1% SDS and exposed to Kodak Biomax film for 4 days at –80°C.

2.3. Electrophoretic Mobility Shift Assay

Nuclear extracts from untreated cells were isolated [26] and both gel shift and super shift assays were run on nondenaturing gels [27] using radiolabeled PPRE as target. A double stranded PPRE was prepared by annealing the sense and antisense-oligonucleotides 5'-CGCGTGACCAAGGACAAAGGTACGTTTC-3' and 5'-TCGAGAACGTGACCTGTTGCCTGGTCG-3', respectively. Probes were created by extending the nucleotides with Klenow enzyme in the presence of [α ³²P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA). As a control for non-specific binding, oligonucleotides containing an androgen response element (ARE) and an estrogen response element (ERE) were used as cold competitor. These sequences are 5'-CTAGCTGTACAGGATGTTCTAGCTACTGTA-

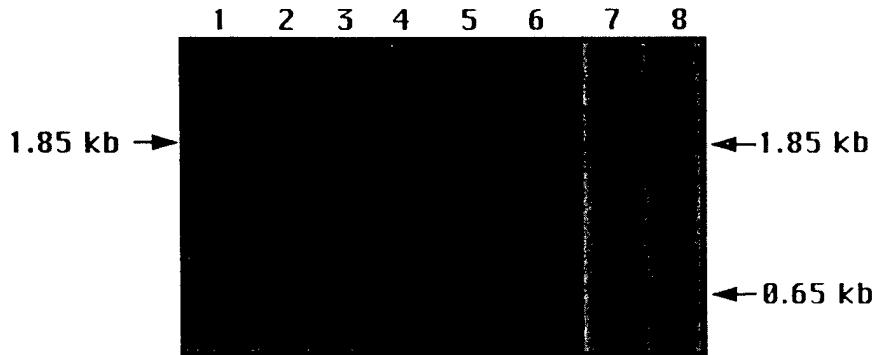


Fig. 1. Northern blot analysis of PPAR in human breast cancer cells. Three micrograms of Poly A⁺ RNA was isolated from MCF-7 cells (lane 2) and run against 30 µg of total RNA from Sunflower (*Helianthus annuus* cv Mammoth) (lane 4) as a negative control. Lane 1 contains size marker and lane 3 was left blank to avoid overlapping bands when a very long exposure was used. On a separate blot, 5 µg total RNA from MCF-7 cells and 5 µg of total RNA from human adipose tissue were run in lanes 5 and 6, respectively. In all cases, a single band of approximately 1.85 kb was seen. Five micrograms of poly (A)⁺ RNA from T47D cells was run from control (lane 7) or cells treated with 10⁻⁸ estradiol (lane 8). Bands of 1.85 and 0.65 kb were observed, both of lesser intensity than MCF7 or adipose tissue.

CAGGATGTTCTC-3' for the sense and 5'-TCGAGA-GAACATCCTGTACAGTAGCTAGAACATCCTG-TACAG-3' for the antisense of the ARE and 5'-CTAGCGGTCACTGTGACCCAAGCTTGGGTC-ACAGTGACCC-3' for the sense and 5'-TCGAGGGT-CACTGTGACCCAAGCTTGGGTCACAGTGACCG-3' for the antisense of the ERE.

For the gel mobility shift analysis, 5 µg of nuclear extract was incubated in a final volume of 21 µl containing 21 mM HEPES (pH 7.9), 75 mM KCl, 0.2 mM EDTA (pH 8.0), 20% glycerol, 0.5 mM PMSF, 24 µM leupeptin, 0.15 µM pepstatin, 0.3 µM aprotinin, 2 µg polydeoxyinosinic-deoxycytidyl acid (Pharmacia, Piscataway, NJ) at 20°C for 15 min. Three hundred femto-moles of labeled probe and between 6 and 24 pmol of competitor (20–80-fold molar excess, respectively) oligonucleotide for displacement analysis was added and incubated for an additional 15 min. The reaction mixture was loaded directly onto a 1.5 mm thick, 4% polyacrylamide gel (37.5:1, acrylamide:bisacrylamide) in 0.5 × TBE buffer and run at 150 V. The gel was dried on a filter paper and exposed to BioMax MR film (Eastman Kodak, Rochester, NY) at -80°C with an intensifying screen. Supershifts were performed as described for the mobility shift assays [27] using specific antibodies (1 µl) which were added during the first incubation period. Two different antibodies were used in supershift assays, a polyclonal raised in rabbit to amino acids 484–498 anti-mPPAR γ 2 (Cat # PA3-820, Affinity Bioreagents) from the C' terminal end of PPAR γ 2 and is highly conserved in mouse, rat and human PPAR γ 1, a and NUC1. A second polyclonal antibody (Cat # PA3-821, Affinity Bioreagents) raised in rabbit against amino acids 294–298 from mouse PPAR γ 2. These residues are completely conserved in mPPAR γ 1. As a control for the specificity of the antibodies used in supershift analysis, 4 µl of non-immune rabbit IgG (ABI) was used.

2.4. Transient Transfection Analysis

Both MCF7 and T47D cells were transiently transfected using the calcium phosphate method [26]. Each well received 10 µg 3 × PPRLuciferase plasmid DNA driven by a minimal TK promoter [10] and 0.33 µg β -galactosidase containing plasmid, constitutively driven by the CMV promoter. Following treatments, cells were lysed in 210 µl lysis buffer and treated according to manufacturer's instructions (Analytical Luminescence Laboratory). Luciferase activity from 40 µl was measured for 10 s (ALL) and 15 µl was used to measure β -galactosidase activity according to the Galacto-Light instructions (Tropix) for 5 s. Luminometry was performed on a Monolight 2010 (ALL) and data reported as the mean \pm S.E.M. fold stimulation above nontreated controls. These values were obtained by dividing the luciferase RLU by the β -galactosidase RLU from each of three separate wells. Each set of treatments was performed in triplicate and the data reported as the mean \pm S.E.M. from three separate experiments.

3. Results

To determine whether PPAR is expressed in breast cancer cells, poly A⁺ mRNA was isolated from both T47D and MCF-7 cells. RNA from sunflower (*Helianthus annuus* cv. Mammoth) and human adipose tissue were employed as negative and positive controls, respectively. Following electrophoretic separation the gel was stained with ethidium bromide to assess loading and quality of the RNA. The blot was probed with a mPPAR γ 2-specific probe created as described. The resulting autoradiogram indicates that MCF-7 cells contain a single 1.85 kb band (Fig. 1, lanes 2 and 5) which

is the same size as that seen in transcripts from human adipose tissue (Fig. 1, lane 6). This is also the size seen in mouse and in human PPAR γ 1 from hematopoietic cells [16]. Greene and co-workers [16] also described a 0.65 kb band (termed PPAR γ 2) which was not seen in MCF7 cells. Five micrograms of total RNA from T47D cells was also subjected to Northern blot analysis and revealed the presence of less intensive bands at 1.85 and 0.65 kb (Fig. 1, lanes 7 and 8). By contrast, no detectable bands were seen in sunflower mRNA (Fig. 1, lane 4) even upon very long exposure (data not shown).

The binding specificity of proteins present in the nuclear extracts of MCF-7 cells was examined by gel shift analysis using a radiolabeled PPRE. As seen in Fig. 2 (lane 2), four specific bands (B, C, D and E) are seen when using nuclear extracts from MCF-7 cells. Using an 80-fold molar excess of cold PPRE was sufficient to compete all of these bands (lane 3) indicating that all four are specifically bound. To confirm the presence of PPAR in the complex seen on the EMSA, further retardation of the complex by specific antibody

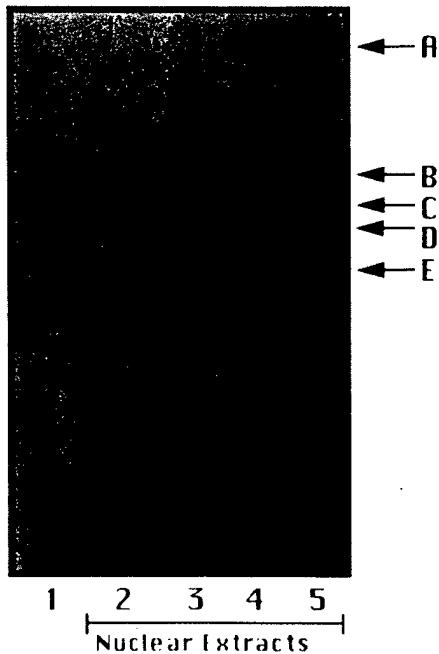


Fig. 2. Electrophoretic mobility shift assay of nuclear extracts from MCF-7 cells using a consensus PPRE. The peroxisomal proliferator response element from the acyl-CoA oxidase promoter of rat was used to design oligonucleotides which were annealed and labeled as described and used as probe. The control (lane 1) contains no extracts, while lanes 2–5 contain 3.5 μ g of nuclear extract. Non-specific binding was assessed by adding 80-fold molar excess cold PPRE (lane 3). Supershift analysis was performed using two different monoclonal antibodies against the mPPAR γ . The first, raised to amino acids 484–498 (PA3-820) was used in lane 4 while the second, against amino acids 294–298 (PA3-821), was used in lane 5. Arrows indicating bands B, C, D and E were specifically competed by cold competitor while band A indicates the supershifted band obtained with either antibody.

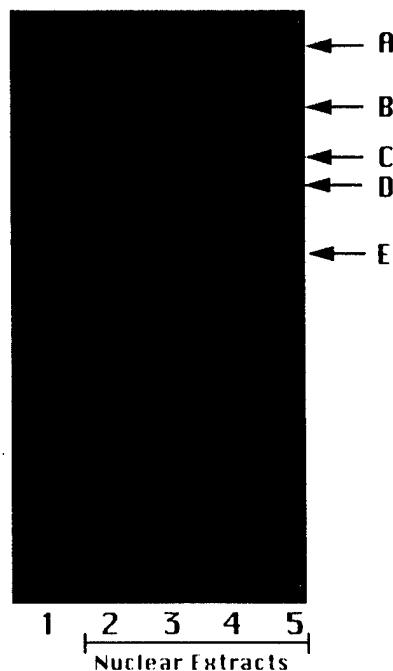


Fig. 3. Electrophoretic mobility shift assay of nuclear extracts from MCF-7 cells. Nuclear extracts from MCF-7 cells were used to test the ability of similar response elements to compete for binding with a consensus peroxisome proliferator response element as a radiolabeled probe. Lane 1 is a no protein control, lanes 2–5 contain 3.5 μ g nuclear protein. Lane 2 lacks competitor and lane 3 contains an 80-fold excess of cold PPRE as competitor. Lanes 4 and 5 contain an 80-fold excess of cold estrogen response element (ERE) or an 80-fold excess of cold androgen response element (ARE), respectively.

ies was attempted. Supershift analysis (Fig. 2, lanes 4 and 5) indicates that antibodies raised to two different portions of mPPAR γ were able to specifically bind resulting in a slower mobility complex (band A). Although it appears that band B is specifically and preferentially supershifted, confirmation of these data must await further analysis and identification of the proteins in the other bands. As a positive control, electrophoretic mobility shift assay and supershift analyses of nuclear extracts from mouse NIH-3T3 cells was shown to recognize and bind a labeled PPRE and this complex was also supershifted using both the 820 and 821 antibodies (data not shown). To demonstrate the specificity of DNA binding, oligonucleotides containing two EREs in tandem or two AREs in tandem were used as a cold competitor against a radiolabeled PPRE. Fig. 3 demonstrates that an 80-fold excess of an ERE (lane 4) or an ARE (lane 5) was not capable of competing for binding with the PPRE. The inability of the ARE or ERE to compete PPRE binding clearly indicates that cross-competition is not taking place between the PPRE and the very similar ERE or the more dissimilar ARE. Together, these data indicate that PPAR is present in the nuclei of MCF-7 cells and is part of a complex capable of recognizing and specifically binding to a PPRE.

Transient transfection analysis was undertaken to determine whether MCF-7 and T47D cells were functionally capable of responding to peroxisomal proliferators by altering the transcriptional regulation of target genes. As independent stimulators of PPAR and RXR, Wy 14,643 (Wy) and 9-cis retinoic acid (RA) were used, respectively. MCF-7 cells were stimulated 18 h while T47D cells were treated for 24 h. Cells treated individually with Wy did not significantly activate transcription of the reporter (Fig. 4). Co-treatment with 9-cis retinoic acid with Wy 14,643, however, significantly raised expression of the reporter above 9-cis retinoic acid alone. These data indicate that both cell types are capable of transcriptionally regulating target genes containing a consensus PPRE when stimulated by a peroxisomal proliferator. A second reporter with a single copy of the DR1 site cloned in front of the SV40 promoter of pGL3 (Promega) was also stimulated by Wy and RA; however, the fold induction was less dramatic due to the presence of the very strong SV40 viral promoter (data not shown).

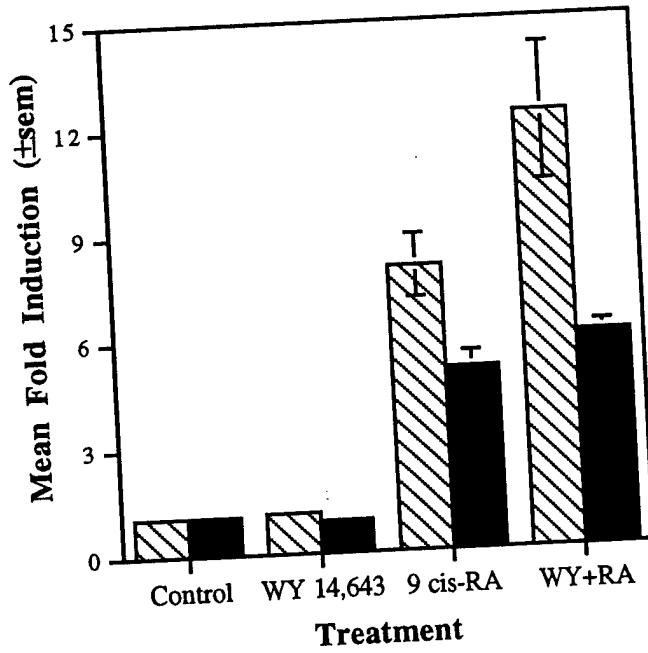


Fig. 4. Peroxisome proliferator-mediated transcriptional regulation by transient transfection analysis in MCF-7 and T47D human breast cancer cells. Both MCF-7 (hatched bars) and T47D cells (solid bars) were cotransfected with a β -galactosidase reporter constitutively driven by a CMV promoter as an internal control for transfection efficiency and a plasmid reporter containing three copies of a PPRE cloned in tandem upstream of a thymidine kinase promoter mediating luciferase expression. Cells were starved in charcoal stripped serum for 7 days prior to transfection and following an overnight recovery were treated for 18 (MCF-7) or 24 h (T47D) with Wy 14,643 at 10^{-6} M, 9-cis retinoic acid at 10^{-6} M or both at 10^{-6} M. Raw luminometric units (RLU) for luciferase were divided by the RLU for β -galactosidase and data reported as the fold induction over control. All treatment groups were performed in triplicate and data reported as the mean \pm S.E.M. from three separate experiments.

4. Discussion

Although intensively studied, the cause and effect relationship between diet and specific cancers, including breast, has been very difficult to draw in humans and remains highly controversial. Furthermore, properly controlled dietary studies in humans are elusive, if not impossible. Although animal models utilizing implanted human cell lines have been used somewhat successfully in the study of fat intake on the invasiveness of implanted cells [4–6], these can never serve as a faithful representation of human disease since the fatty acid mobilization and transport pathways differ significantly. Therefore, the goal of these analyses was to provide a possible molecular mechanism whereby dietary fats might affect breast tumors. Since PPARs are activated by dietary fatty acids, and high-fat diets may play a role in breast disease, this study was designed to determine if human breast cancer cell lines contained a functional response to peroxisomal proliferators.

Peroxisomal proliferators are non-genotoxic carcinogens which exert their effects on cells through their interactions with PPARs, themselves members of the nuclear receptor family. Activators of PPARs are structurally diverse and their effects differ in a tissue-specific manner. It is now clear that the molecular mechanism behind these pleiotropic effects is due to the presence of multiple isoforms of this receptor which are themselves expressed in a tissue-specific manner. Using Northern blot analysis we have been able to detect a single hybridizing species at 1.85 kb, precisely the size of hPPAR γ 1 [16], in MCF7 and both the 1.85 and 0.65 kb bands in T47D cells. The relative intensities between the MCF7 and T47D cells indicates that MCF7 cells express higher levels of PPAR mRNA than do T47D cells. Although high stringency conditions were used in these Northern blots, the true identification of the receptor and determination of the subtype(s) must await isolation and sequence analysis of a cDNA clone. These studies are currently underway in the laboratory.

Electrophoretic mobility shift assays and supershift assays indicate that nuclear proteins from human breast cancer cells are present that can specifically bind to a PPRE. The fact that as many as four bands were present in these assays may indicate that multiple proteins or dimer combinations are capable of recognizing this motif. Although others have implicated the estrogen receptor and RXR β in mediating PPAR action [28], it is clear more work must be done to address the significance of these findings and the role of other proteins in mediating PPRE binding and thus transcription [29]. The ability of anti-PPAR antibodies to further retard the mobility of a labeled probe indicate that PPAR is indeed present in this complex and participates in the recognition of specific hormone responsive elements of target genes. Although the binding pattern

of proteins to the PPRE is complex, it is clear that a PPAR participates in PPRE binding. Additional studies will be necessary to delineate the nature of each complex seen in gel shift analysis and determine the functional significance of each. The ability of these antibodies to supershift proteins from the NIH-3T3 cells further confirms this conclusion. Furthermore, the fact that neither an ERE, which differs by a single base from the PPRE half-site, nor an ARE were capable of competing with the PPRE for binding demonstrates the high degree of specificity proteins from breast cancer cells have for the PPRE.

Since PPAR has been demonstrated to heterodimerize with RXR α , itself a receptor for 9-cis retinoic acid, we have attempted transient transfection analysis with ligands for both PPAR and RXR alone and in combination. The fact that 9-cis retinoic acid alone is nearly as effective as its co-stimulation with WY 14,643 suggests that other peroxisome proliferators may be more effective or that the media contains components capable of activating PPAR and thus maintaining it at some level of activation. It is also possible that the endogenous production of prostaglandins maintains PPAR in a constant state of activation in these cells. These questions are critical to our understanding the activation of peroxisomal proliferation in breast cancer cells and is currently being examined in the lab. Although the physiological significance of a functional PPAR in human breast cancer cells remains to be determined, these data demonstrate that MCF-7 cells are a useful model to study the effects of peroxisome proliferators such as fatty acids in the human breast.

Acknowledgements

We would like to acknowledge Evan Simpson, M. Dod Michael and Ron Evans for plasmids used in these studies. We also would like to thank Douglas Stocco, M. Dod Michael and Anna Lauber-Biason for critical discussion of this work. This work was supported by the Greenville Hospital System/Clemson University Cooperative Research and Education program.

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Email mce@mednet.swmed.edu

9 December, 1999

Dr. Michael Kilgore
Department of Microbiology and Molecular Medicine and
GHS/CU Biomedical Cooperative
124 Long Hall
Clemson University
Clemson, SC 29634-1909
United States

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Differential Transcriptional Activation of Peroxisome Proliferator-Activated Receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 Cells.

Sudha R. Thoennes^a, Patricia L. Tate^{a,b}, Thomas M. Price^b, Michael W. Kilgore^{a,b*}

^aDepartment of Microbiology and Molecular Medicine and ^bGreenville Hospital /Clemson University Cooperative Research and Education Program, 124 Long Hall, Clemson University, Clemson, SC 29634-1909, USA

*Corresponding Author:

Michael W. Kilgore, PhD
Department of Microbiology and Molecular Medicine and
GHS/CU Cooperative Research and Education Program
124 Long Hall
Clemson University
Clemson, SC 29634-1909
Tel 864.656.1859
Fax 864.656.1127
Email MKilgor@Clemson.Edu

Keywords: PPAR γ , transcription, breast cancer, dietary fats, Omega-3 fatty acids, Omega-6 fatty acids

ABSTRACT

While the role of dietary fats in breast cancer remains controversial, the recent cloning of peroxisome proliferator-activated receptor gamma (PPAR γ), a nuclear hormone receptor, from human breast cancer cells lines provides a potential molecular link. Several fatty acids from four classes of dietary fats were tested for their ability to mediate the transcriptional activity of PPAR γ in MCF-7 and MDA-MB-231 cells using growth media with minimal serum. Whereas omega-3 fatty acids inhibit transactivation of PPAR γ to levels below control, omega-6, monounsaturated and saturated fatty acids stimulate the activity of the transcriptional reporter. These studies indicate that individual fatty acids differentially regulate the transcriptional activity of PPAR γ by selectively acting as agonists or antagonists. Furthermore, the transcriptional activation of PPAR γ correlates with cell proliferation in MCF-7 cells. Understanding the effects of individual fats on breast cancer cells and PPAR γ transactivation could provide important new insights into the epidemiology of breast cancer and the role of dietary fat.

INTRODUCTION

Breast cancer is the leading cause of the cancer deaths among American women [1]. In 1997, 180,200 women were diagnosed with breast cancer and approximately 43,900 women died of this disease in the United States [2]. In contrast 1 in 40 Chinese women will develop breast cancer and this difference can not be accounted for by genetic factors [3]. Although epidemiological analysis suggests a correlation between high-fat diets and breast cancer in humans [4-12], controversy remains. Animal studies, however, have provided convincing evidence of this link and clearly demonstrate a correlation between dietary fats, types of fats ingested and mammary tumors [13-19].

It has been suggested that ω -6 fatty acids, which are high in Western diets, might be associated with higher risk of breast cancer incidences [5,19]. By contrast, populations whose fat intake is primarily ω -3 fatty acids have a lower incidence of breast cancer [17] and ω -3 fatty acids inhibit growth and metastatic potential of human cells in animal models [15,16,20]. The results from animal studies suggest that both the amount [21-23] and type [19,24-31] of fats consumed play a role in the susceptibility, growth and metastatic potential of both chemically induced and surgically implanted tumors.

The studies presented in this report were performed to determine whether differential effects of fatty acids on MCF-7 and MDA-MB-231 cells might be mediated by PPAR γ . Peroxisome proliferator-activated receptors are members of the nuclear hormone receptor family that play an important regulatory role in adipogenesis and lipid metabolism [32,33]. These transcription factors control the expression of genes encoding enzymes in lipid metabolic pathways [32,33]. Three genes encoding PPAR have been identified in mammals and are termed PPAR α , β/δ and γ [32,33]. While the physiological ligand remains unresolved, both synthetic and natural ligands have been reported [32-37]. Recently we reported that several human breast cancer cell lines express PPAR γ and contain a functional response to synthetic peroxisome proliferators [38]. This finding has been confirmed by others in humans [39,40] as well as mice [41]. In this report we demonstrate that individual fatty acids selectively function as agonists or antagonist of PPAR γ in MCF-7 and MDA-

MB-231 cells and the activation of PPAR γ correlates with an increase in cell proliferation in MCF-7 cells. This model enables us to begin to examine the molecular mechanism whereby individual components from a complex diet might alter growth and development of breast cancer.

MATERIALS AND METHODS

Chemicals

All fatty acids, Calcium chloride, HeBS reagent chemicals (NaCl, KCl, Na₂HPO₄.2H₂O), Me₂SO, indomethacin and Bovine Serum Albumin (BSA) were purchased from Sigma chemical Co. (St. Louis, MO). Concentrated stocks of free fatty acids including the monounsaturated fatty acids, (MUFAs) and the polyunsaturated fatty acids (PUFAs) were prepared either in EtOH (Apper Alcohol, Kentucky) or Me₂SO and final concentrations were made by dilutions with the culture medium. Ly 171,883 (Eli Lilly, Indianapolis, IN) was dissolved in EtOH and the final concentrations of EtOH and Me₂SO in all conditions was 0.1%.

Cell Culture

MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in the Dulbecco's modified Eagle's medium (DMEM) or improved MEM (IMEM), respectively (GibCo) containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. Cells were gradually adapted to TCH® serum replacement medium (Celox Laboratories, Inc., St. Paul, MN) supplemented with 0.5% FBS (HyClone, Logan, Utah) for MCF-7 or without serum for MDA-MB-231 cells. All media lacked phenol red. Cells were routinely grown in T-75 flasks (Corning) and transferred to 6-well plates (Corning) two days prior to transfection. Cells were transfected at \approx 40-50 % confluence and not allowed to go through more than 20 passages to minimize genetic drift inherent in culture system.

Reporter Plasmid Construction and Transient Transfection Assays

The DR1-SV40-LUC, was constructed by annealing and ligating complementary 27 bp oligonucleotides containing single copy of underlined DR1 response element (5'-CGCGTGACCAGGTCAAAGGTCA CGTTC) into the unique Mlu1-Xho1 site of the pGL3-Promoter vector (Promega). The 3xPPRE-TK-luciferase construct was described previously [42]. MCF-7 and MDA-MB-231 cells were transfected using the calcium phosphate method [43]. Each

well received 10 µg 3x PPRE-luciferase plasmid DNA driven by a minimal TK promoter and 0.33 µg β-galactosidase containing plasmid, constitutively driven by the CMV promoter. Following treatments, cells were lysed in 200 µl lysis buffer and treated according to manufacturer's instructions (Analytical Luminescence Laboratory). Luciferase activity from 40 µl of lysate was measured for 10 sec (ALL) and 15 µl lysate was used to measure β-galactosidase activity according to the Galacto-Light instructions (Tropix) for 5 sec on a Monolight 2010 (ALL). The relative luminescent values for each well were derived by dividing the inducible luciferase values by the constitutive β-galactosidase values. Stock solutions of omega-3 PUFA, omega-6 PUFA and monounsaturated fatty acids were made in absolute ethanol, and the working solutions were made by dilutions in TCH medium. Stock solutions of saturated fatty acids were prepared in Me₂SO (DMSO) and the working solutions were made in TCH medium containing BSA at a final concentration of 0.2%. Each set of treatments was performed in triplicate in three independent experiments. Statistical analysis is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison analysis and confidence limits were set at p≤0.05. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

Cell Proliferation Assay

Proliferation was determined by measuring the incorporation of tritiated thymidine into cellular DNA. Thirty hours after treatment 1.0 µCi [³H] thymidine was added to each well and cells incubated for an additional 6 hrs. Each well was washed four times in PBS and the adherent cells were lysed in 500 µl of 1x trypsin-EDTA and 500 µl of a lysing solution (2 N NaOH, 6.82 mM, N-lauroyl sarcosine and 10 µM EDTA). Lysates were individually transferred into 15 ml centrifuge tubes (Fisher) containing 100µl of 0.5% Phenol red (Sigma) and 150 µl of formaldehyde, 37% w/w (Fisher). Samples were adjusted to a pH of 7.4 as determined by colormetric analysis against a know standard using HCl and NaOH. Samples from each culture well were passed through a

0.45 μ m filter (Millipore) using a 12 filter Millipore manifold. Filters were air-dried, placed in vials with 8 ml of Scintiverse scintillation fluid and counted on a Beckman LS6500. Each set of treatments were performed in replicates of 5 in three independent experiments. The experimental design is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison analysis and confidence limits were set at $p \leq 0.05$. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

RESULTS

To determine whether PPAR γ activated the DR-1 and 3xPPRE constructs, both were tested in MCF-7 cells. The synthetic peroxisome proliferator LY 171,883 stimulated reporter activity in cells transfected with either the PPRE or the DR-1 equally (data not shown). Furthermore, the null vectors lacking the DR-1 or the three PPREs were unresponsive indicating the cloned response elements mediated these effects (data not shown).

Next we examined the ability of individual fatty acids to function as ligands and mediate the transcriptional regulation of PPAR γ . MCF-7 cells were transiently transfected with the 3xPPRE-TK-luciferase and treated individually with omega-3 PUFA, omega-6 PUFA, monounsaturated fatty acids or saturated fatty acids. In all cases, the fatty acid concentrations used in these studies were those shown to be maximally effective in this culture system. This was determined over a range of fatty acid concentrations. Of the omega-3 PUFAs, linolenic acid (LnA, C18:3 ω 3), eicosapentaenoic acid (EpA, C20:5 ω 3), docosahexaenoic acid (DhA, C22:6 ω 3) and docosapentaenoic acid (DpA, C22:5 ω 3) were tested. The results in figure 1 demonstrate that omega-3 PUFA inhibit the transcriptional activity of PPAR γ to levels below control. The strongest inhibition was observed by EPA (100 μ M), which inhibited reporter activity to 66% ($p \leq 0.001$) relative to control. Linolenic acid resulted in 80% reporter activity at 10 nM ($p \leq 0.002$) relative to control. DhA also inhibited PPAR γ activity to 89% of control at 10 μ M concentration ($p \leq 0.02$). By contrast, DpA did not

inhibit the transcriptional activity of PPAR γ . Similar results were obtained in another set of experiments in which MCF-7 cells were transfected with DR1-SV40-LUC reporter construct and treated with omega-3 PUFAs (data not shown).

Three omega-6 PUFAs, linoleic acid (LaA, C18:2 ω 6), arachidonic acid (ArA, C20:4 ω 6) and γ -linolenic acid, (γ -LnA, C18:3 ω 6) were tested (figure 2). Gamma-Linolenic acid (200 μ M) stimulated reporter activity 1.63 fold induction ($p\leq 0.005$) while linoleic acid (250 μ M) resulted in a 1.57 fold induction ($p\leq 0.005$) and arachidonic acid (250 μ M) produced a 1.52 fold induction ($p\leq 0.005$). Similar results were seen in MDA-MB-231 cells where linoleic acid (250 μ M) significantly inducted reporter activity of the 3xPPRE-luciferase reporter 4.2 fold over control ($p\leq 0.001$, data not shown).

Figure 3 demonstrates the effect of two monounsaturated fatty acids (MUFAs), oleic acid (C18:1 ω 9) and petroselinic acid (C18:1 ω 12), on PPRE-mediated reporter activity in MCF-7 cells. Oleic acid (350 μ M) increased reporter activity 1.24 fold ($p\leq 0.05$) whereas petroselinic acid (150 μ M) stimulated reporter activity 1.85 fold ($p\leq 0.001$).

Five saturated fatty acids with increasing chain lengths, caprylic (C10:0), palmitic (C16:0), stearic (C18:0), arachidonic (C20:0) and lignoceric acid (C24:0) were tested (Figure 4). Both caprylic (10 μ M) and palmitic acid (10 μ M) weakly stimulated reporter activity 1.15 fold ($p\leq 0.02$) and 1.11 fold, respectively. Lignoceric acid treatment (50 μ M) stimulated reporter activity 1.25 over control ($p\leq 0.05$). A 1.2 fold induction ($p\leq 0.05$) was seen with arachidonic acid treatment (100 μ M) and stearic acid (250 μ M) increased reporter activity 1.4 fold ($p\leq 0.002$).

Using the same concentrations reported in the transcriptional assays, the effects of linoleic and linolenic acids were examined for their ability to induce proliferation (figure 5). Estrogen, in the form of 17 β -estradiol, a known mitogen in MCF-7 cells, significantly increased cell proliferation as did linoleic acids ($p\leq 0.001$ and $p\leq 0.005$, respectively). By contrast, linolenic acid significantly inhibited cell proliferation to levels below control ($p\leq 0.01$).

DISCUSSION

In an attempt to further clarify the role of individual dietary components on the physiology of human breast cancer cells, we have tested a variety of fatty acids for their ability to modulate the transcriptional activity of PPAR γ . Three important observations have been made as a consequence of these studies. First, individual fatty acids appear to selectively function as agonists or antagonists of PPAR γ in MCF-7 cells. Although variability exists between individual fatty acids within a class, clear differences exist between the classes of fatty acids themselves. These data suggest that transactivation of PPAR γ may be one mechanism whereby individual fatty acids can mediate cell-specific function by modulating gene expression. The fact that reporter activity was also stimulated in MDA-MB-231 cells with linoleic acid indicates this is not a response specific to MCF-7 cells and may be more generally applicable to other breast cancer cells. Secondly, while we have previously demonstrated that MCF-7 cells express high levels of mRNA, these data suggest that PPAR γ is constitutively transactivated in MCF-7 cells. This is supported by the observation that ω -3 fatty acids such as linolenic acid inhibits the activity of the transcriptional reporter to levels below that of control. This suggests that linolenic acid can compete with some endogenous ligand thus inhibiting basal activity. Thirdly, linoleic acid, an agonist of PPAR γ function, increases the rate of cell proliferation while linolenic acid treatment, a PPAR γ antagonist, reduces proliferation. This is in contrast to troglitazone, a synthetic agonist of PPAR γ , which has been reported to inhibit proliferation of 21MT cells [40] and induce apoptosis when used in combination with all-*trans*-retinoic acid in MCF-7 cells [39]. Evans and co-workers, however, have demonstrated that activators of PPAR γ increase the incidence of colorectal tumors and polyp formation indicating that transactivation of PPAR γ may indeed result in tumor formation [44]. In support of our proliferation studies animals fed linolenic acid have fewer tumors and a reduced metastatic potential when compared to animals maintained on diets rich in linoleic acid [29,45]. The differences between the in vitro and in vivo data could be a function of cell-specific responses or due to differences in the ligand-receptor interactions. Although these data do not prove a cause and effect

relationship between transactivation of PPAR γ and the control of cell cycle, the mechanism of this effect in MCF-7 cells clearly warrants further investigation.

In NIH 3T3 cells, a mouse adipose stromal cell line, transfection with PPAR γ is both necessary and sufficient to set in motion the differentiation into an adipocyte. The presence of PPAR γ in human breast cancer cells has led to the speculation that it's transactivation could be used therapeutically to induce the re-differentiation of malignant cells into a benign state [40]. Here we report that MCF-7 cells express PPAR γ and the receptor exists in some continuous state of transcriptional activation, yet these cells do not differentiate into adipocytes. This suggests that the transactivation of PPAR γ in MCF-7 cells sets in motion a program unique from that seen in adipose stromal cells. Clearly, it will be important to examine the genes regulated by PPAR γ in human breast cancer cells to understand the functional significance of these observations. Additionally, further studies will be necessary to determine the molecular mechanisms of tissue-specific responses.

A question critical to determining the role PPAR γ plays in human breast cancer is to assess its expression in normal ductile epithelia as well as primary and metastatic tumors. Gimble and co-workers reported that PPAR γ is expressed in ductile epithelia of virgin mice and rats [41]. However, PPAR γ is not expressed during lactation nor in mammary tumors induced by 7,12-dimethylbenz(a)anthracene [41]. Furthermore, NmuMG, a normal mouse epithelial line, expresses PPAR γ but its expression is not inducible by peroxisome proliferators. In contrast to the rodent model, PPAR γ is expressed in several human cancer cell lines including T-47-D, MDA-MB-231, SK-BR3, ZR-75-1 and BT-20 cells [38,40] and a functional response to peroxisome proliferators has been demonstrated in MCF-7 [38] and 21MT cells [40]. Finally, Spiegelman and coworkers have shown that PPAR γ is expressed in both primary breast tumors and in lung sections of patients with metastatic tumors [40]. The expression of PPAR γ in both benign and malignant cells leaves open the possibility that peroxisome proliferators could play a role in both normal and cancerous tissue.

The role of lipid in breast disease has been the subject of intense debate and, more recently, intensive investigation. The identification of a functional response to peroxisome proliferators in a wide variety of breast cancer cell lines and the ability of fatty acids to selectively mediate the transactional activity of PPAR γ lends further support for a direct role of dietary fatty acids in human breast cancer. Clearly, the functional significance of these findings will require further investigation.

FIGURE LEGENDS

Figure 1. Effects of omega-3 fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Cells were transiently transfected with reporter plasmid and treated with vehicle alone (control), eicosapentaenoic acid (EPA), linolenic acid (LnA), eicosahexaenoic acid (DhA) or docosapentaenoic acid (DpA) for 18-24 hrs. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($p\leq 0.05$).

Figure 2. Effects of omega-6 fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Cells were transfected with the reporter plasmid and treated with vehicle alone (control), or linoleic acid (LaA), arachidonic acid (ArA) or γ -linolenic acid (γ -LnA), for 18-24 hrs. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($p\leq 0.05$).

Figure 3. Effects of monounsaturated fatty acids on the transcriptional activity of PPAR γ in MCF-7 cells. Following transfection, cells were treated for 18-24 hrs with vehicle alone (control), oleic or petroselinic. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($p\leq 0.05$).

Figure 4. Effects of saturated fatty acids on the transcriptional activity of PPAR γ MCF-7 cells. MCF-7 cells were transfected with the reporter plasmid and treated with vehicle alone (control), caprylic, palmitic, stearic, arachidic or lignoceric acid, as described, for 18-24 hrs. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($p\leq 0.05$).

Figure 5. Effects of 17 β -estradiol (E₂), linoleic and linolenic acid on MCF-7 cell proliferation. Cells were plated at equal densities and treated for 30 hrs with physiological concentrations of estradiol or of fatty acids at the same concentrations used in transfection experiments. Each well received ³[H]-thymidine plus treatments for an additional 6 hrs. Following ANOVA, data are plotted as the treatment means (\pm one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($p \leq 0.05$).

Acknowledgments

This work was supported by a grant from the Breast Cancer Research Program (DAMD17-97-1-7248 to M.W.K.) and grants from the Greenville Hospital/Clemson University Cooperative Research and Education Program (to M.W.K. and T.M.P.). We would like to thank Regina Brierley for technical assistance, Dr. Wen Chen for critical review and helpful comments and Dr. Herman Senter for statistical analysis.

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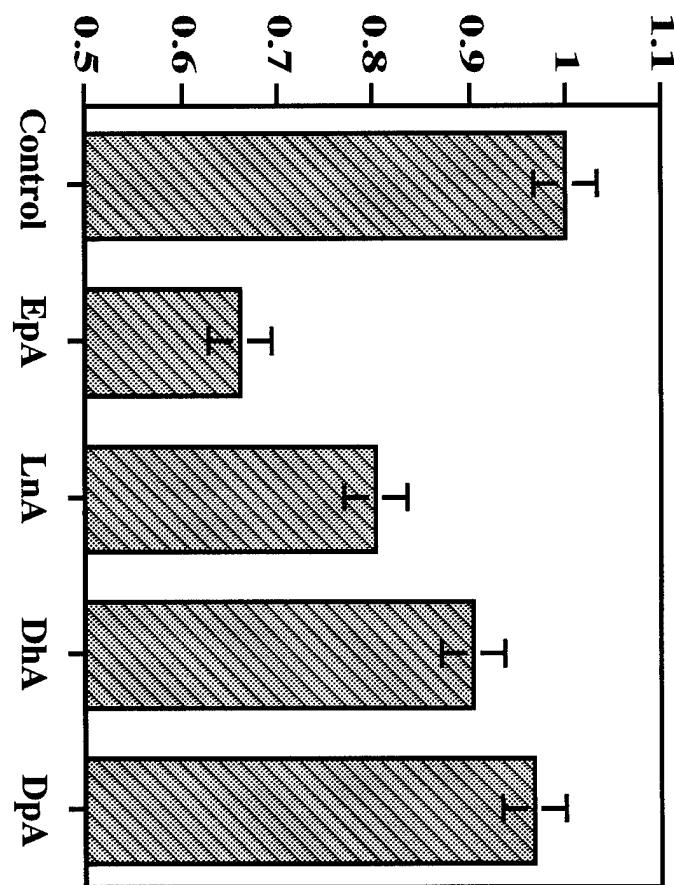
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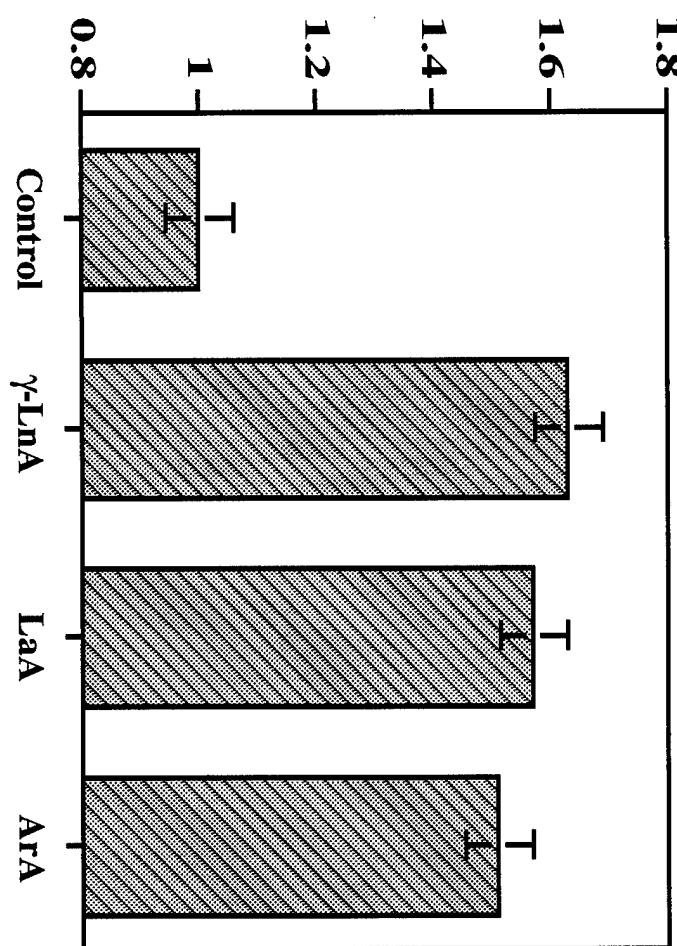
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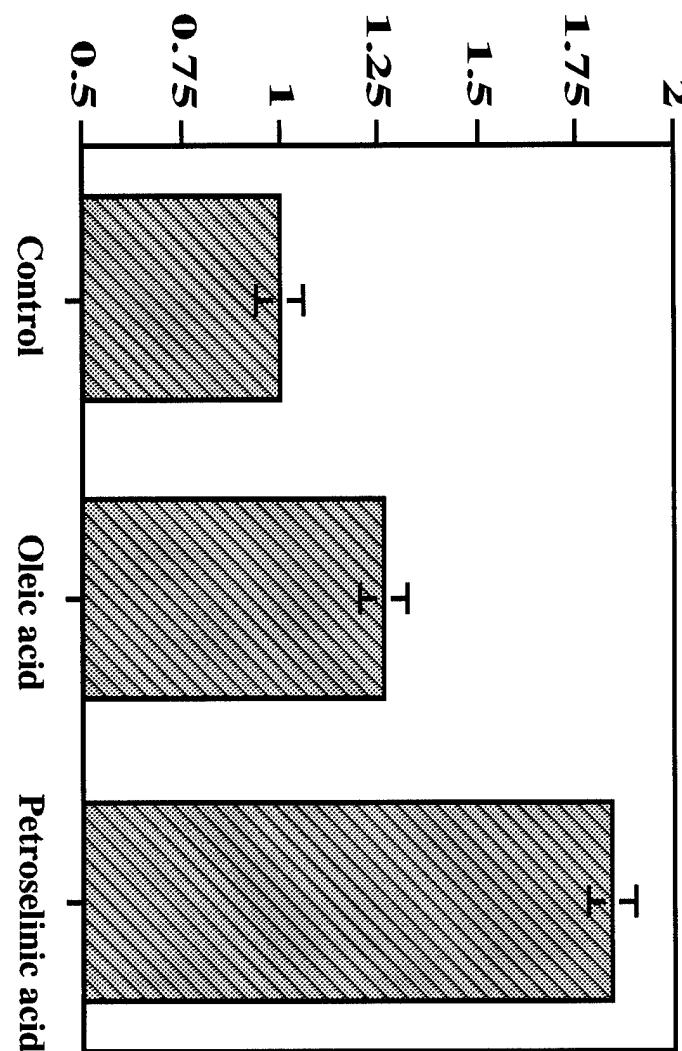
Mean Fold Induction
(\pm critical value for comparison/2)



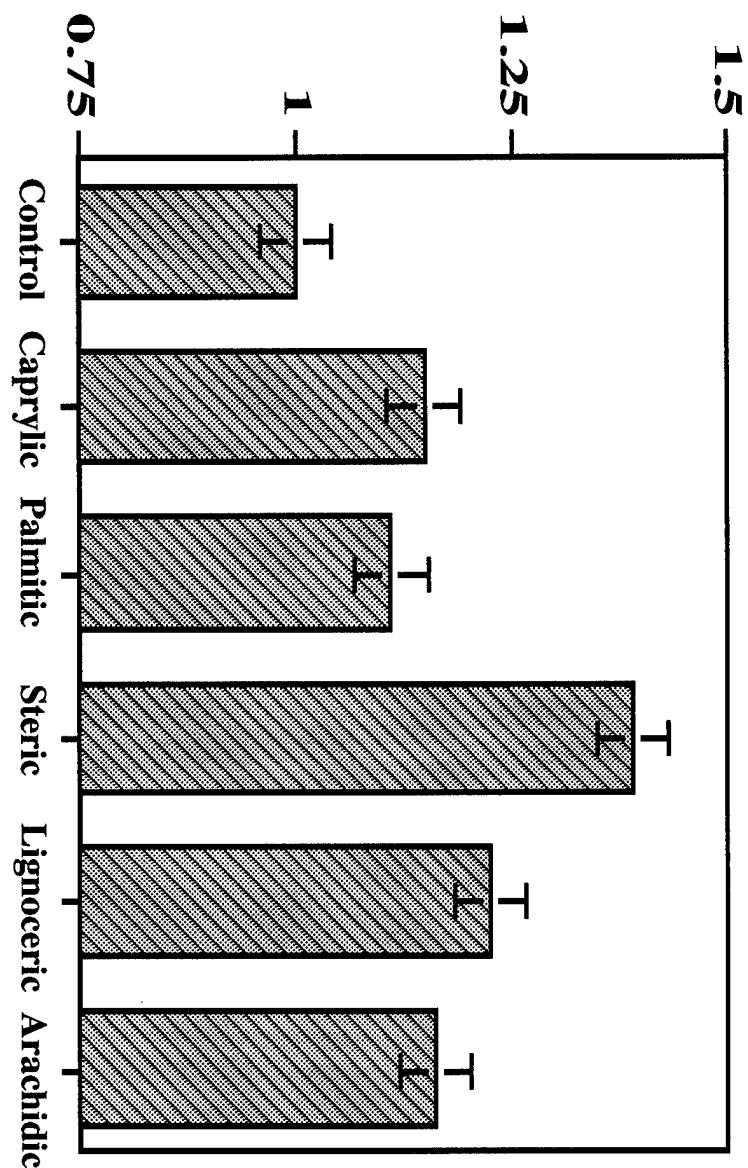
Mean Fold Induction
(\pm critical value for comparison/2)



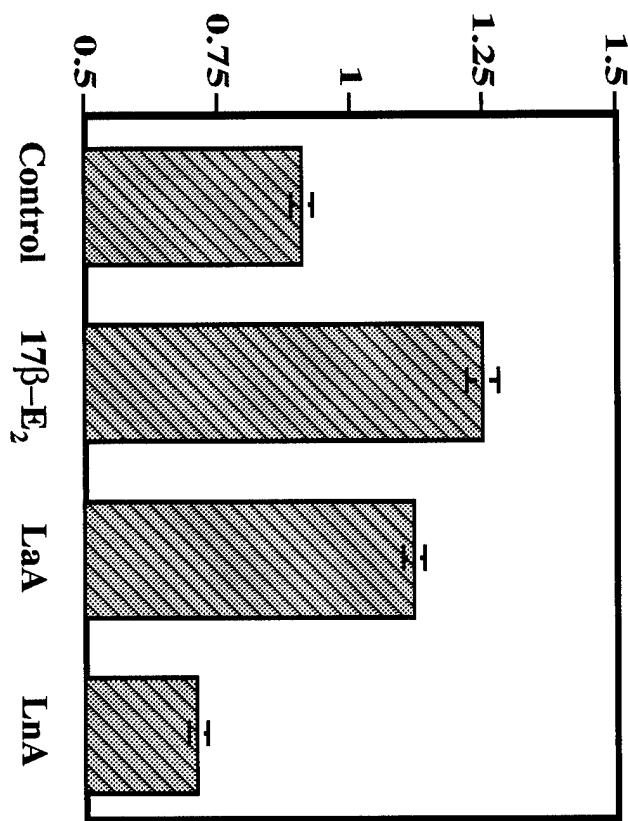
Mean Fold Induction
(\pm critical value for comparison/2)



Mean Fold Induction
(\pm critical value for comparison/2)



**Mean ^3H -Thymidine
Incorporation cpm* 10^{-3}**



7th Annual South Carolina Research Conference

Wild Dunes Isle of Palms, SC, January 1999

Peroxisome Proliferator Activated Receptor: Isolation and Possible Role in Human Breast Cancer Cells.

Sudha Rai, Eiichi Sengoku, Patricia L. Tate, Thomas M. Price and Michael W. Kilgore.
Greenville Hospital/ Clemson University Cooperative Research and Education Program
Department of Microbiology and Molecular Medicine & Biological Sciences, Clemson
University, Clemson, SC 29634.

Epidemiological and experimental investigations have indicated that several dietary and endocrine-related factors can, if not induce, modulate the growth of breast cancer. Recently, a ligand activated nuclear receptor, termed peroxisome proliferator activated receptor (PPAR) has been cloned and shown to be regulated by, among other factors, high- fat diet. Studies conducted in our lab have shown that MCF-7 and T47D human breast cancer cells contain a functional peroxisomal response (Mol Cell Endo. 129,1997). Here we report that individual fatty acids activate PPAR-mediated transcription in breast cancer cells, using what may be a unique form of PPAR gamma2. We have constructed a cDNA library from MCF-7 breast cancer cells and isolated several clones utilizing a PPAR gamma2 specific probe. After screening and sequencing, we found a clone, C1, which contains an insert 5' of the consensus ATG start site of transcription (#L40904). Recently, PPAR from a fetal rabbit type II pneumonocyte cDNA library has been isolated and sequenced. This rabbit PPAR contains sequence which matches the insert seen in the C1 clone. After identifying the PPAR gamma2 isoform in MCF-7 cells, we set about to examine the effect of individual fatty acids on the activation of PPAR. Initially, we optimized the cell growth conditions to maximize MCF-7 cells' responsiveness to treatment using a variety of serum treatments and serum supplements. Since serum itself contains endogenous lipids, it can lead to false positive cell response to fatty acid treatment. We have examined the effects of individual omega-3 and omega-6 fats, alone and in combination with indomethacin, on transactivation of PPAR in transient transfection analysis. These studies suggest that individual fatty acids play a physiological role in the regulation of PPAR in human breast cancer which, in turn, might provide invaluable information for therapeutic management of this devastating disease.

80th Annual meeting of the Endocrine Society, June 1998

New Orleans, LA

ROLE OF DIETARY FATS IN PPAR-MEDIATED CARCINOMA IN MCF-7 HUMAN BREAST CANCER CELL LINE. Sudha Rai*, Patricia L. Tate, Thomas M. Price, Michael W. Kilgore Department of Microbiology and Molecular Medicine and the Greenville Hospital System/Clemson University Biomedical Cooperative, Clemson University, Clemson, SC 29634

Epidemiologic, clinical and animal studies suggest that consumption of essential fatty acids may modulate breast cancer risk and tumor growth. The type of fat consumed in Western and Eastern countries, as well as its amount, are emerging as an issue of major importance. Western diets are mainly constituted of omega-6 polyunsaturated fatty acids whereas eastern diets are rich in omega-3 fats. Treatment of rodents with diet high in polyunsaturated fatty acids (PUFA) resulted in profound proliferation of peroxisomes in hepatic parenchyma cells. Two theories have been proposed to explain this tumorigenesis: (a) a biochemical hypothesis whereby fatty acid accumulation inside the cell stimulates peroxisomal proliferation, (b) the response is a receptor mediated event. The latter theory got support by the recent cloning of a ligand mediated nuclear receptor, Peroxisome Proliferator-Acivated Receptor (PPAR). We have cloned a member of PPAR from MCF-7 breast cancer cells and demonstrated the presence of a functionally responsive receptor capable of mediating the transcriptional activation of target genes in these cells. We have established a molecular model based on transient transfection analysis to examine the role of individual fats in tumorigenesis. A reporter construct containing three copies of the peroxisome proliferator response element (PPRE) upstream of TK promoter was used in transactivation studies. After testing a number of diverse synthetic peroxisome proliferators in MCF-7, the PPAR form appeared to be of gamma isotype. At the next step, four classes of dietary fats, namely, omega-3 PUFA, omega-6 PUFA, monounsaturated fats and saturated fats have been tested, alone and in combination with the cyclooxygenase inhibitor indomethacin. Tumor growth and progression have been linked to the end-product of omega-6 fatty acid (in particular arachidonic acid) metabolism. Arachidonic acid is metabolised through cyclooxygenase and lipoxygenase pathways to eicosanoids, prostaglandins, thromboxanes and leukotrienes. Animal studies have shown that omega-3 fats serve as competitive inhibitors in these pathways and generate eicosanoids which may act as tumor suppressors. These studies support a potential role of omega-3 fatty acids in inhibiting the growth of MCF-7 human breast cancer cells. The objective of this study is to find the molecular basis of the link between ingestion of dietary fats and growth of human breast cancer cells which might be of immense value in therapeutic management of this devastating disease.

81st Annual meeting of the Endocrine Society, June 1999

San Diego, CA

Signal Cross-talk between Peroxisome Proliferator-activated receptor (PPAR) and Estrogen receptor (ER) in human Breast Cancer Cells, Xin Wang*, Patricia L. Tate*, Thomas M. Price and Michael W. Kilgore*, Department of Microbiology and Molecular Medicine, GHS/CU Biomedical Cooperative, Clemson University, Clemson, SC 29634

While both family history and lifetime exposure to estrogen are related to an increased breast cancer risk, considerable epidemiological data also suggest there is also a link between high fat diets. Due to the complexity of this relationship, however, this link remains highly controversial. Animal studies suggest dietary fatty acid may have a direct role in the growth and metastasis of human breast cancer independent of their systemic effects. Recently, PPAR has been identified as a ligand activated nuclear receptor. It can be activated directly by fatty acids and mediates transcription from its response element (PPRE). PPAR and its heterodimeic partner, the retinoid X receptor (RXR), have been shown to bind several kinds of estrogen response element (ERE), suggesting that some EREs may also function as a PPAR response elements (PPRE) dependent upon cell and promoter context. To determine whether ER and PPAR interact in breast cancer cells, we have established a molecular model using transient transfection analysis. We have constructed plasmids with three consensus PPRE or an ERE driving the expression of firefly luciferase as a reporter of transcriptional activation. To examine the signal cross-talk between the PPAR and the ER, we employ MDA-MB-231 cells, which are ER negative. Reportes constructs are co-transfected with and without ER α or ER β expression vectors. Cells are subsequently treated by 17 β -estradiol (E $_2$), BRL48,482, a synthetic PPAR ligand, or E $_2$ and BRL in combination. These analysis show that in the absence of the ER both E $_2$ or BRL can inhance the transcriptional regulation using a PPRE, but the presence of ER α or ER β can inhibit this response. Using the same conditions, both E $_2$ and BRL can upregulate the ERE-mediated reorter in the presence of either ER α or β but not in the absence of exogenous ER. As we know, the ER belongs to a large family of nuclear hormone receptors. The differences in the amino-terminal regions of ER α and ER β can promote specific differences in transcriptioinal activity of its receptor. These data indicate that significant cross-talk may exist between PPAR and ER and raises the possibility that dietary fats may alter ER-media responses as well as transactivating PPAR directly and could help to explain the role of dietary fats in the etiology of breast cancer.

Key Words: PPAR, ER, Breast cancer

8th Annual South Carolina Research Conference

Wild Dunes Isle of Palms, SC, January 1999

Signal Cross-talk between Peroxisome Proliferator-Activated Receptor and Estrogen Receptor in human Breast Cancer Cells

X Wang, PL Tate, TM Price and MW Kilgore

Department of Microbiology and Molecular Medicine

GHS/CU Biomedical Cooperative, Clemson University, Clemson, SC 29634-1909.

While both family history and lifetime exposure to estrogen are related to an increased breast cancer risk, considerable epidemiological data suggest there is also a link between high fat diets and the occurrence of breast cancer. Due to the complexity of this relationship, however, this link remains highly controversial. Animal studies suggest dietary fatty acids may have a direct role in the growth and metastasis of human breast cancer independent of their systemic effects. Recently, Peroxisome Proliferator-Activated Receptor (PPAR) has been identified as a ligand activated nuclear receptor. PPAR can be activated directly by fatty acids and mediate transcription from its response element (PPRE). PPAR and its heterodimeric partner, the retinoid X receptor (RXR), have been shown to bind several kinds of estrogen response element (ERE), suggesting that some EREs may also function as a PPRE dependent upon cell and promoter context. To determine whether ER and PPAR interact in breast cancer cells, we have established a molecular model using transient transfection analysis. We have constructed a plasmids with three consensus PPRE and an ERE driving the expression of firefly Luciferase as a reporter of transcriptional activation. To examine the signal cross talk between the PPAR and the ER, we employ MDA-MB-231 cells, which are ER negative. Report constructs are co-transfected with and without ER α or ER β expression vectors. Cells are subsequently treated with 17 β -estradiol (E $_2$), BRL 48,482, a synthetic PPAR ligand, or E $_2$ and BRL in combination. The analysis show that in the absence of the ER both E $_2$ or BRL can enhance the transcriptional regulation of genes containing a PPRE, but the presence of ER α or ER β can inhibit this response. Using the same conditions, both E $_2$ and BRL can upregulated the expression of the ERE reporter gene in the presence of either ER α or β . These data indicate that significant cross talk may exist between the PPAR and ER and raises the possibility that dietary fats may alter ER-mediated responses as well as transactivating PPAR directly. These data could help to explain the role of dietary fats in the etiology of breast cancer.

9th Annual South Carolina Research Conference
Wild Dunes Isle of Palms, SC, January 2000

**REGULATION OF PEROXISOME PROLIFERATOR-ACTIVATED
RECEPTOR GAMMA (PPAR γ) GENE EXPRESSION IN MCF-7 AND MDA-MB-
231 HUMAN BREAST CANCER CELLS.**

EVELINA R. LOGHIN^a and MICHAEL W. KILGORE^{a,b,*}

^aDEPARTMENT OF BIOLOGICAL SCIENCES

^bDEPARTMENT OF MICROBIOLOGY and MOLECULAR MEDICINE, and

*GREENVILLE HOSPITAL/CLEMSON UNIVERSITY COOPERATIVE RESEARCH
and EDUCATION PROGRAM,

132 LONG HALL, CLEMSON UNIVERSITY, CLEMSON,
SC 29634-2001, USA

ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand activated transcription factors, which include the nuclear hormone receptors. Ligands include long chain fatty acids and antidiabetic thiazolidinedione drugs. Transactivation of PPAR has been associated with hepatic and colorectal cancer development. To determine whether PPAR functions as a molecular link between dietary fat consumption and breast cancer we are examining its functional relevance in human breast cancer cells. Thus far three distinct PPARs, alpha, beta and gamma, have been identified, each encoded by a separate gene with distinct tissue distribution. In man three different subtypes of PPAR gamma have been identified, the consequence of multiple promoter usage. Previous experiments performed in our lab suggest that both MCF-7 and MDA-MB-231 breast cancer cells express PPAR gamma and are functionally responsive to peroxisome proliferators(PP) including fatty acids. Based on transient transfection experiments with PPAR gamma antisense expression constructs PPAR appears to be constitutively transcribed, translated and transactivated at some basal level on both MCF-7 and MDA-MB-231. Furthermore transient transfection with 5' flanking region of PPAR γ 1, PPAR γ 2, and PPAR γ 3, driving the expression of a luciferase reporter construct, suggests that PPAR γ 1 is the primary regulator of the basal PPAR expression in both MCF-7 and MDA-MB-231. Although the functional significance of PPAR expression and transactivation in breast cancer cells is currently unknown this may provide insights into dietary means of prevention and potential target for therapeutic intervention.

9th Annual South Carolina Research Conference
Wild Dunes Isle of Palms, SC, January 2000

**A Dominant Negative Mutant of PPAR γ Reduces PPRE-mediated Reporter Activity
in MCF-7 Human Breast Cancer Cells.**

Susan Peirce^a and Michael W. Kilgore^{a++}

^aDepartment of Biological Sciences; 132 Long Hall, Clemson University, Clemson, SC 29634-20001, USA.

⁺⁺Department of Microbiology and Molecular Medicine; Greenville Hospital/Clemson University Cooperative Research Program; 132 Long Hall, Clemson University, Clemson, SC 29634-20001, USA.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear receptor superfamily of transcription factors. It is highly expressed in a wide variety of tissues and plays a role in adipocyte differentiation, and the formation of atherosclerotic plaques and colorectal tumors. We have shown that PPAR γ is expressed and is functionally responsive in human breast cancer cells. Recently, a dominant negative mutant form of PPAR γ was identified from a patient presenting with a severe form of diabetes, and the altered genotype was localized to a single C to T mutation resulting in a proline to lysine transition in the ligand binding domain. The mutant is able to bind RXR (the retinoid receptor heterodimeric partner of PPAR), but not the antidiabetic ligand rosiglitazone BRL49653, a synthetic peroxisome proliferator. We have examined the functional role of this mutant in human breast cancer cells by first creating a cDNA subclone of P467L using PCR techniques. A human breast cancer cell line, MCF-7, was transiently transfected in a dual system using a second vector containing luciferase and renilla as reporter genes 3' to the PPAR responsive element, the PPRE. Here we demonstrate that P467L reduces PPRE-mediated reporter activity. Further, as expected, P467L is completely unresponsive to induction with BRL48482. Finally, using a proliferative assay measuring incorporation of BrdU as an index of DNA synthesis, it appears that this inhibition of expression does not result from a reduction in cell proliferation. With this mutant in hand, we will be able to examine the functional significance of PPAR expression in breast cancer cells.